**PCT** WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 15/86, 7/04, 7/01, C07K 14/035 — A1

(11) International Publication Number: WO 98/04726

(43) International Publication Date: 5 February 1998 (05.02.98)

(21) International Application Number: PCT/GB97/02017

(22) International Filing Date: 25 July 1997 (25.07.97)

(30) Priority Data:
9615794.6 26 July 1996 (26.07.96) GB

(71) Applicant *(for all designated States except US)*: MEDICAL RESEARCH COUNCIL [GB/GB]; 20 Park Crescent, London W1N 4AL (GB).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: COFFIN, Robert, Stuart [GB/GB]; UCL Medical School, Division of Pathology, Dept. of Molecular Pathology, The Windeyer Building, 46 Cleveland Street, London W1P 6DP (GB). LATCHMAN, Seymour, David [GB/GB]; UCL Medical School, Division of Pathology, Dept. of Molecular Pathology, The Windeyer Building, 46 Cleveland Street, London W1P 6DP (GB). MACLEAN, Alasdair, Roderick [GB/GB]; MRC Virology Unit, Church Street, Glasgow G11 5JR (GB). BROWN, Suzanne, Moira [GB/GB]; Neurovirology Research Laboratories, Institute of Neurological Sciences, Southern General Hospital NHS Trust, Glasgow G51 4TF (GB).

(74) Agent: WOODS, Geoffrey, Corlett; J.A. Kemp & Co., 14 South Square, Gray's Inn, London WC1R 5LX (GB).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

**Published**

*With international search report.*

*Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.*

(54) Title: HSV STRAIN LACKING FUNCTIONAL ICP27 AND ICP34.5 GENES

(57) Abstract

The present invention provides a herpes simplex virus strain which lacks a functional ICP34.5 gene and a functional ICP27 gene. It also provides the use of a herpes simplex virus strain which lacks a functional ICP34.5 gene and a functional ICP27 gene in the treatment of disorders of, or injuries to, the nervous system of a mammal.

*FOR THE PURPOSES OF INFORMATION ONLY*

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

| | |
|---|---|
| AL | Albania |
| AM | Armenia |
| AT | Austria |
| AU | Australia |
| AZ | Azerbaijan |
| BA | Bosnia and Herzegovina |
| BB | Barbados |
| BE | Belgium |
| BF | Burkina Faso |
| BG | Bulgaria |
| BJ | Benin |
| BR | Brazil |
| BY | Belarus |
| CA | Canada |
| CF | Central African Republic |
| CG | Congo |
| CH | Switzerland |
| CI | Côte d'Ivoire |
| CM | Cameroon |
| CN | China |
| CU | Cuba |
| CZ | Czech Republic |
| DE | Germany |
| DK | Denmark |
| EE | Estonia |
| ES | Spain |
| FI | Finland |
| FR | France |
| GA | Gabon |
| GB | United Kingdom |
| GE | Georgia |
| GH | Ghana |
| GN | Guinea |
| GR | Greece |
| HU | Hungary |
| IE | Ireland |
| IL | Israel |
| IS | Iceland |
| IT | Italy |
| JP | Japan |
| KE | Kenya |
| KG | Kyrgyzstan |
| KP | Democratic People's Republic of Korea |
| KR | Republic of Korea |
| KZ | Kazakstan |
| LC | Saint Lucia |
| LI | Liechtenstein |
| LK | Sri Lanka |
| LR | Liberia |
| LS | Lesotho |
| LT | Lithuania |
| LU | Luxembourg |
| LV | Latvia |
| MC | Monaco |
| MD | Republic of Moldova |
| MG | Madagascar |
| MK | The former Yugoslav Republic of Macedonia |
| ML | Mali |
| MN | Mongolia |
| MR | Mauritania |
| MW | Malawi |
| MX | Mexico |
| NE | Niger |
| NL | Netherlands |
| NO | Norway |
| NZ | New Zealand |
| PL | Poland |
| PT | Portugal |
| RO | Romania |
| RU | Russian Federation |
| SD | Sudan |
| SE | Sweden |
| SG | Singapore |
| SI | Slovenia |
| SK | Slovakia |
| SN | Senegal |
| SZ | Swaziland |
| TD | Chad |
| TG | Togo |
| TJ | Tajikistan |
| TM | Turkmenistan |
| TR | Turkey |
| TT | Trinidad and Tobago |
| UA | Ukraine |
| UG | Uganda |
| US | United States of America |
| UZ | Uzbekistan |
| VN | Viet Nam |
| YU | Yugoslavia |
| ZW | Zimbabwe |

# HSV STRAIN LACKING FUNCTIONAL ICP27 AND ICP34.5 GENES

## Field of the Invention

The present invention relates to mutant herpes simplex virus strains which have inactivating mutations rendering them non-pathogenic. It also relates to the use of such mutant HSV strains in gene therapy and in methods of assaying for gene function.

## Background to the invention

Herpes simplex virus (HSV) has often been suggested as a suitable vector for the nervous system due to its neurotrophic lifestyle and its ability to remain in neurons for the lifetime of the cell. However wild type HSV is highly pathogenic and must like most viral vectors be disabled in some way. The pathogenic effects of HSV result from lytic infection with the virus and therefore the use of HSV as a vector requires the development of strains carrying mutations that disrupt the lytic cycle whilst allowing the establishment of asymptomatic latent infections. HSV vectors have previously been produced and tested *in vivo* by the deletion of the essential immediate early (IE) gene ICP4 (Dobson *et al.*, 1990 and Chiocca *et al.*, 1990), which must be complemented for growth in culture. ICP4 is required for transcriptional activation of the viral early and late genes in lytic infection. Thus, a virus lacking this gene can readily establish latent infection of cells but cannot grow lytically.

Mutations have also been made in non-essential genes such as the IE gene ICP0, the IE gene ICP6, tyrosine kinase (TK), US5 or VMW65, all of which are required for full pathogenicity *in vivo* but are dispensable for growth in culture (reviewed by Coffin and Latchman, 1996). These types of mutation provide the added advantage that the deletion need not be complemented for growth in culture which has been shown previously to occasionally result in reversion of the non-pathogenic phenotype to a wild-type phenotype by homologous recombination between the virus and the complementing sequences in the cell-line during growth. However in each of these cases, mutation of the non-essential gene does not completely prevent virus replication since high titre inoculation will overcome the block to replication *in vivo*.

We have tested, as vectors, HSV mutant strains deleted for the ICP34.5 gene - the so-called neurovirulence factor - which is absolutely required for neurovirulence *in vivo*, but is again unnecessary for growth in culture (Chou *et al.*, 1990). Mutations in ICP34.5 provide a subtle mechanism by which HSV can be disabled. ICP34.5 is thought to prevent the usual host response to a productive infection in neurons, which results (in the absence of ICP34.5) in cell death and thus the limitation of the infection to initially infected cells. ICP34.5 is thought to over-ride this response and allow full lytic replication to occur. Thus in the absence of ICP34.5, if a disabled virus were ever to re-establish a productive infection for whatever reason, the ICP34.5 mutation would ensure that the protective host response limited virus replication to a small number of cells.

To test the possibility that ICP34.5 deleted herpes viruses might be developed as vectors for the nervous system, we inserted a lacZ construct into a non-essential gene (UL43) of the HSV-1 ICP34.5 deletion mutant strain 1716, and inoculated mice via the footpad route (for delivery to dorsal root ganglia (DRGs)) and intracranially. LacZ activity (as assayed by X-gal staining), could be seen in a limited number of cells in the DRGs and brain respectively (unpublished observations). These results indicate that HSV strains carrying inactivating mutations in ICP34.5 are suitable for use as gene-delivery vectors. ICP34.5 deletion mutants could thus provide the basis for further development as novel and safe gene-delivery vectors for the nervous system.

However, it is unlikely that viruses carrying a single defect will be considered safe enough for eventual human use. Added safety and the possibility of higher titre inoculation might be achieved by the deletion of an essential IE gene providing an absolute block to replication (and which must thus be complemented in culture), together with ICP34.5. Previously HSV vectors disabled by removal of essential IE genes and used *in vivo* have been deleted for ICP4 as this single deletion absolutely prevents replication and provides the greatest reduction in the levels of other HSV gene products. However the other IE genes (ICP0, 27, 22 and 47) are still produced and of these the essential ICP27 is highly cytotoxic probably due to its secondary role of preventing the splicing of pre-mRNAs in favour of translation from the mainly unspliced herpes RNAs. We therefore speculated that removal of ICP27 (to be complemented in culture) might produce a safer and less cytotoxic vector system when combined with deletions in ICP34.5. While a number of ICP27 deletion mutant viruses have been produced and used, for example, to study herpes gene regulation *in vitro* and the

effects of ICP27 on the host cell (Reef Hardy and Sandri-Goldin, 1994 and Rice and Knipe, 1990), none has reportedly been tested as a vector for gene delivery to the nervous system *in vivo*. Furthermore, none of the ICP27 deletion mutant viruses carry a mutation in ICP34.5

## Summary of the Invention

This invention relates to mutant herpes simplex virus strains which have been disabled for use as gene delivery vectors by the functional inactivation of both ICP34.5 and ICP27. Such HSV strains can be used, for example, for delivering therapeutic genes in methods of treatment of diseases of, or injuries to, the nervous system, including Parkinson's disease, spinal injury or strokes, or diseases of the eye, heart or skeletal muscles, or malignancies. The present invention also relates to methods for studying the function of genes in mammalian cells, for example in identifying genes complementing cellular dysfunctions, or studying the effect of expressing mutant genes in wild-type or mutant mammalian cells. The methods of the present invention may be used in particular for the functional study of genes implicated in disease.

We have now surprisingly found that HSV strains carrying inactivating mutations in both ICP34.5 and ICP27 genes exhibit greatly improved levels of expression of heterologous genes compared to virus strains carrying mutations in ICP34.5 alone. These doubly-mutated strains are also safer than strains carrying mutations in ICP27 alone. We have also shown that an additional inactivating mutation in ICP4 and an inactivating mutation in VMW65, which abolishes its transcriptional-activation activity, reduces further the toxicity of the viral strains of the invention. Thus, the viral strains of the present invention are not only safer than previous strains, but also offers high levels of expression of heterologous genes.

Accordingly the present invention provides a herpes simplex virus strain which lacks a functional ICP34.5 gene and a functional ICP27 gene. Preferably, the HSV strain of the invention further lacks a functional form of other IE genes, more preferably the IE gene ICP4. Inactivation of the essential IE ICP4 gene prevents viral replication and provides the greatest reduction in the levels of other HSV gene products. The HSV strain of the invention preferably also lacks a functional *vhs* gene and/or a functional VMW65 gene due to a mutation in said VMW65 gene that abolishes its transcriptional-activation activity. In a particulary preferred embodiment of the present invention, the HSV strain lacks a functional

ICP34.5 gene, a functional ICP27 gene, a functional ICP4 gene and a functional VMW65 gene due to a mutation in said VMW65 gene which abolishes its transcriptional-activation activity.

The invention further provides an HSV strain of the invention which carries a heterologous gene. The term heterologous gene is intended to embrace any gene not found in the HSV genome. The heterologous gene may be any allelic variant of a wild-type gene, or it may be a mutant gene. Heterologous genes are preferably operably linked to a control sequence permitting expression of said heterologous gene in mammalian cells, preferably cells of the central or peripheral nervous system, or cells of the eye, heart or skeletal muscle, more preferably cells of the central or peripheral nervous system. The HSV strain of the invention may thus be used to deliver a heterologous gene to a mammalian cell where it will be expressed. Such vectors are useful in a variety of applications, for example, in gene therapy, or *in vitro* assay methods or for the study of HSV gene regulation.

The heterologous gene preferably encodes a polypeptide of therapeutic use, including polypeptides that are cytotoxic or capable of converting a precursor prodrug into a cytotoxic compound.

The invention further provides herpes simplex virus strains of the invention, carrying a heterologous gene, for use in the treatment of humans and animals. For example, such HSV strains may be used in the treatment of diseases of, or injury to, the nervous system, including Parkinson's disease, spinal injury or strokes or disease of the eye, heart or skeletal muscle, or malignancies.

The HSV strains of the present invention may also be used in methods for studying the function of genes in mammalian cells, for example in identifying genes complementing cellular dysfunctions, or studying the effect of expressing mutant genes in wild-type or mutant mammalian cells. The methods of the present invention may be used in particular for the functional study of genes implicated in disease.

The invention also provides a method for producing a herpes simplex virus of the present invention, said method comprising modifying the ICP34.5 and ICP27 genes of a herpes simplex virus so as to inactivate them functionally. The method of the invention may further comprise modifying a second IE gene, preferably the ICP4 gene, so as to inactivate it functionally, and/or modifying the VMW65 gene so to inactivate functionally its transcriptional-activation activity.

Detailed Description of the Invention

A. Viral Strains

The HSV strains of the invention may be derived from, for example, HSV-1 or HSV-2 strains, or derivatives thereof, preferably HSV-1. Derivatives include inter-type recombinants containing DNA from HSV-1 and HSV-2 strains. Derivatives preferably have at least 80% sequence homology to either the HSV-1 or HSV-2 genomes, more preferably at least 90%, even more preferably 95%. Other derivatives which may be used to obtain the HSV strains of the present invention include strains that already have mutations in either ICP34.5 or ICP27, for example strain 1716 (MacLean *et al.*, 1991), strains R3616 and R4009 (Chou and Roizman, 1992) and R930 (Chou *et al.*, 1994) all of which have mutations in ICP34.5, and d27-1 (Rice and Knipe, 1990) which has a deletion in ICP27. Viral strains that have deletions in other HSV genes may also be conveniently used, for example strain d120 which has a deletion in ICP4 (DeLuca *et al.*, 1985) or strain d92 which has deletions in both ICP27 and ICP4 (Samaniego *et al.*, 1995). Use of these strains will reduce the number of steps required to produce the mutant HSV strains of the present invention.

The terminology used in describing the various HSV genes is as found in Coffin and Latchman, 1996.

B. Complementing cell lines

The virus of the invention is propagated on a cell line expressing ICP27, for example V27 cells (Rice and Knipe, 1990), 2-2 cells (Smith *et al.*, 1992) or B130/2 cells (see Example 1), preferably B130/2 cells.

ICP27-expressing cell lines can be produced by co-transfecting mammalian cells, for example the Vero or BHK cells, with a vector, preferably a plasmid vector, comprising a functional HSV ICP27 gene capable of being expressed in said cells, and a vector, preferably a plasmid vector, encoding a selectable marker, for example neomycin resistance. Clones possessing the selectable marker are then screened further to determine which clones also express functional ICP27, for example on the basis of their ability to support the growth of

ICP27$^-$ HSV strains, using methods known to those skilled in the art (for example as described in Rice and Knipe, 1990).

Cell lines which do not allow reversion of an ICP27$^-$ mutant HSV strain to a strain with functional ICP27 are produced as described above, ensuring that the vector comprising a functional ICP27 gene does not contain sequences that overlap with (i.e. are homologous to) sequences remaining in the ICP27$^-$ mutant virus.

Where HSV strains of the invention comprise inactivating modifications in other essential genes, for example ICP4, complementing cell lines will further comprise a functional HSV gene which complements the modified essential gene in the same manner as described for ICP27. For example in the case of HSV strains comprising mutations in both ICP27 and ICP4, a cell line expressing both ICP27 and ICP4 is used (such as E26 cells (Samaniego *et al.*, 1995) or B4/27 cells (see Example 3), preferably B4/27 cells).

Cells expressing ICP27 and ICP4 are produced by transfecting mammalian cells, for example Vero or BHK cells, with similar vectors as those used for production of ICP27-expressing cell lines, i.e. expressing ICP27 and a selectable marker, together with a third vector, preferably a plasmid vector, comprising a functional ICP4 gene capable of being expressed in said cells. Clones possessing the selectable marker are then screened further to determine which clones also express ICP27 and ICP4, for example on the basis of their ability to support growth of ICP27 and ICP4 mutant HSV strains, again using methods known to those skilled in the art (for example as described in Samaniego *et al.*, 1995).

C. Methods of mutation.

The ICP34.5 and ICP27 genes may be rendered functionally inactive by several techniques well known in the art. For example, they may be rendered functionally inactive by deletions, substitutions or insertions, preferably by deletion. Deletions may remove portions of the genes or the entire gene. Inserted sequences may include the heterologous genes described below.

Mutations are made in the HSV strains by homologous recombination methods well known to those skilled in the art. For example, HSV genomic DNA is transfected together with a vector, preferably a plasmid vector, comprising the mutated sequence flanked by homologous HSV sequences. The mutated sequence may comprise deletions, insertions or

substitutions, all of which may be constructed by routine techniques. Insertions may include selectable marker genes, for example lacZ, for screening recombinant viruses by, for example, β-galactosidase activity.

Mutations may also be made in other HSV genes, for example IE genes such as ICP0, ICP4, ICP6, ICP22 or ICP47, preferably ICP4, VMW65 or *vhs*. In the case of the VMW65 gene, the entire gene is not deleted since it encodes an essential structural protein, but a small inactivating insertion is made which abolishes the ability of VMW65 to activate transcriptionally IE genes (Ace *et al.*, 1989).

D. Heterologous genes

The mutant HSV strains of the invention may be modified to carry a heterologous gene, that is to say a gene other than one present in the HSV genome. The term "gene" is intended to cover at least sequences which are capable of being transcribed, optionally with some or all of 5' and/or 3' transcribed but untranslated flanking sequences naturally associated with the translated coding sequence. It may optionally further include the associated transcriptional and/or translational control sequences normally associated with the transcribed sequences. The heterologous gene may be inserted into the HSV genome at any location provided that the virus can still be propagated, which may require the use of a cell line carrying another HSV essential gene (as described in B.) if the heterologous gene is inserted into an essential gene. For example, if the heterologous gene is inserted into the ICP4 gene of the mutant HSV strain, then a cell-line expressing both ICP27 and ICP4 would be needed. The heterologous gene is preferably inserted into the region of the ICP27 mutation as in the unlikely event that the mutation is repaired by recombination with a wild-type virus, the repair would remove the inserted heterologous gene.

The heterologous gene may be inserted into the HSV genome by homologous recombination of HSV strains with, for example, plasmid vectors carrying the heterologous gene flanked by HSV sequences. The heterologous gene may be introduced into a suitable plasmid vector comprising HSV sequences using cloning techniques well-known in the art.

The transcribed sequence of the heterologous gene is preferably operably linked to a control sequence permitting expression of the heterologous gene in mammalian cells, preferably cells of the central and nervous system. The term "operably linked" refers to a

juxtaposition wherein the components described are in a relationship permitting them to function in their intended manner. A control sequence "operably linked" to a coding sequence is ligated in such a way that expression of the coding sequence is achieved under conditions compatible with the control sequence.

The control sequence comprises a promoter allowing expression of the heterologous gene and a signal for termination of transcription. The promoter is selected from promoters which are functional in mammalian, preferably human, cells. The promoter may be derived from promoter sequences of eukaryotic genes. For example, it may be a promoter derived from the genome of a cell in which expression of the heterologous gene is to occur, preferably a cell of the mammalian central or peripheral nervous system. With respect to eukaryotic promoters, they may be promoters that function in a ubiquitous manner (such as promoters of α-actin, tubulin) or, alternatively, a tissue-specific manner (such as promoters of the genes for pyruvate kinase). They may also be promoters that respond to specific stimuli, for example promoters that bind steroid hormone receptors. Viral promoters may also be used, for example the Moloney murine leukaemia virus long terminal repeat (MMLV LTR) promoter. The HSV LAT promoter, and promoters containing elements of the LAT promoter region, may be especially preferred because there is the possibility of achieving long-term expression of heterologous genes during latency.

In addition, any of these promoters may be modified by the addition of further regulatory sequences, for example enhancer sequences. Chimeric promoters may also be used comprising sequence elements from two or more different promoters described above, for example an MMLV LTR/LAT fusion promoter (Lokensgard *et al.*, 1994) or promoters comprising elements of the LAT region.

The heterologous gene may encode, for example, proteins involved in the regulation of cell division, for example mitogenic growth factors including neurotrophic growth factors (such as brain-derived neurotrophic factor, glial cell derived neurotrophic factor, NGF, NT3, NT4 and NT5, GAP43 and), cytokines (such as α-, β- or γ-interferon, interleukins including IL-1, IL-2, tumour necrosis factor, or insulin-like growth factors I or II), protein kinases (such as MAP kinase), protein phosphatases and cellular receptors for any of the above. The heterologous gene may also encode enzymes involved in cellular metabolic pathways, for example enzymes involved in amino acid biosynthesis or degradation (such as tyrosine hydroxylase), purine or pyrimidine biosynthesis or degradation, and the biosynthesis or

degradation of neurotransmitters, such as dopamine, or protein involved in the regulation of such pathways, for example protein kinases and phosphatases. The heterologous gene may also encode transcription factors or proteins involved in their regulation, for example members of the Brn3 family (including Brn-3a, Brn-3b and Brn-3c) or pocket proteins of the Rb family such as Rb or p107, membrane proteins (such as rhodopsin), structural proteins (such as dystrophin) or heat shock proteins such as hsp27, hsp65, hsp70 and hsp90.

Preferably, the heterologous gene encodes a polypeptide of therapeutic use. For example, of the proteins described above, tyrosine hydroxylase can be used in the treatment of Parkinson's disease, rhodopsin can be used in the treatment of eye disorders, dystrophin may be used to treat muscular dystrophy, and heat shock proteins can be used to treat disorders of the heart. Polypeptides of therapeutic use may also include cytotoxic polypeptides such as ricin, or enzymes capable of converting a precursor prodrug into a cytotoxic compound for use in, for example, methods of virus-directed enzyme prodrug therapy or gene-directed enzyme prodrug therapy. In the latter case, it may be desirable to ensure that the enzyme has a suitable signal sequence for directing it to the cell surface, preferably a signal sequence that allows the enzyme to be exposed on the exterior of the cell surface whilst remaining anchored to cell membrane. Suitable enzymes include bacterial nitroreductase such as *E. coli* nitroreductase as disclosed in WO93/08288 or carboxypeptidase, especially carboxypeptidase CPG2 as disclosed in WO88/07378. Other enzymes may be found by reference to EP-A-415731. Suitable prodrugs include nitrogen mustard prodrugs and other compounds such as those described in WO88/07378, WO89/10140, WO90/02729 and WO93/08288 which are incorporated herein by reference.

E. Administration

The mutant HSV strains of the present invention may thus be used to deliver therapeutic genes to a human or animal in need of treatment. Delivery of therapeutic genes using the mutant HSV strains of the invention may be used to treat for example, Parkinson's disease, disorders of the nervous system, spinal injury, strokes or malignancies, for example gliomas.

One method for administered gene therapy involves inserting the therapeutic gene into the genome of the mutant HSV strain of the invention, as described above, and then

combining the resultant recombinant virus with a pharmaceutically acceptable carrier or diluent to produce a pharmaceutical composition. Suitable carriers and diluents include isotonic saline solutions, for example phosphate-buffered saline. The composition may be formulated for parenteral, intramuscular, intravenous, subcutaneous, intraocular or transdermal administration.

The pharmaceutical composition is administered in such a way that the mutated virus containing the therapeutic gene for gene therapy, can be incorporated into cells at an appropriate area. For example, when the target of gene therapy is the central or peripheral nervous system, the composition could be administered in an area where synaptic terminals are located so that the virus can be taken up into the terminals and transported in a retrograde manner up the axon into the axonal cell bodies via retrograde axonal transport. The pharmaceutical composition is typically administered to the brain by stereotaxic inoculation. When the pharmaceutical composition is administered to the eye, sub-retinal injection is typically the technique used.

The amount of virus administered is in the range of from $10^4$ to $10^8$ pfu, preferably from $10^5$ to $10^7$ pfu, more preferably about $10^6$ pfu. When injected, typically 1-2 µl of virus in a pharmaceutically acceptable suitable carrier or diluent is administered.

The routes of administration and dosages described are intended only as a guide since a skilled practitioner will be able to determine readily the optimum route of administration and dosage for any particular patient and condition.

F. Assay Methodologies

The mutant HSV strains of the invention can also be used in methods of scientific research. Thus, a further aspect of the present invention relates to methods of assaying gene function in mammalian cells, either *in vitro* or *in vivo*. The function of a heterologous gene could be determined by a method comprising:

(a) introducing said heterologous gene into a mutant HSV strain of the invention;

(b) introducing the resulting HSV strain into a mammalian cell line; and

(c) determining the effect of expression of said heterologous gene in said mammalian cell-line.

For example, the cell-line may have a temperature-sensitive defect in cell division. When an HSV strain comprising a heterologous gene according to the invention is introduced into the defective cell-line and the cell-line grown at the restrictive temperature, a skilled person will easily be able to determine whether the heterologous gene can complement the defect in cell division. Similarly, other known techniques can be applied to determine if expression of the heterologous gene can correct an observable mutant phenotype in the mammalian cell-line.

This procedure can also be used to carry out systematic mutagenesis of a heterologous gene to ascertain which regions of the protein encoded by the gene are involved in restoring the mutant phenotype.

This method can also be used in animals, for example mice, carrying so-called "gene knock-outs". A wild-type heterologous gene can be introduced into the animal using a mutant HSV strain of the invention and the effect on the animal determined using various behavioural, histochemical or biochemical assays known in the art. Alternatively, a mutant heterologous gene can be introduced into either a wild-type or "gene knock-out" animal to determine if disease-associated pathology is induced. An example of this is the use of genes encoding prions to induce Creutzfeld-Jacob and other prion-type diseases in the central nervous system of rodents. Other disease models may include those for Alzheimer's disease, motor neurone disease or Parkinson's disease.

Thus, the methods of the present invention may be used in particular for the functional study of genes implicated in disease.

The invention will be described with reference to the following Examples, which are intended to be illustrative only and not limiting.

## EXAMPLES

### Example 1 - Production of mutant viruses

Viruses

ICP27 deletion mutants were produced by homologous recombination of plasmid pΔMNlacZ with wild-type HSV strain 17+ DNA and also with strain 1716 DNA (MacLean *et al.*, 1991) to generate viruses zΔMN:+ and zΔMN:16 deleted for ICP27 and ICP27 together with ICP34.5 respectively, and each having a lacZ gene driven by the moloney murine leukaemia virus long terminal repeat (MMLV LTR) promoter (Shinnick *et al.*, 1981) replacing the entire oding sequence of ICP27 (and also non-essential genes UL55 and 56). Nucleotide numbers refer to the HSV-1 strain 17+ sequence (Genbank no. HE1CG).

Viruses were generated and stocks prepared by growth on ICP27 complementing BHK cell line B130/2 described below. pΔMnlacz was produced by deleting a NotI/XmnI fragment from the EcoRI B fragment of the HSV-1 genome cloned into pACYC184 (NBL), to leave a fragment which includes the gene for ICP27 and flanking sequences (HSV-1 strain 17+ nts 11095-118439). A pair of MluI fragments encoding the entire ICP27 coding sequence together with the non-essential genes UL55 and 56 (nts 113273-116869) were then removed by digestion with MluI and religation (to give plasmid pΔMN) and replaced by the insertion of an MMLV LTR/lacZ cassette into the MluI site as an NheI/PstI fragment from pJ41acZ after treatment with T4 DNA polymerase, giving plasmid pΔMnlacz. pJ41acZ was produced by insertion of the lacZ gene from pCH110 (Pharmacia) as a BamHI/HindIII fragment into the KpnI site of pJ4 (Morgenstern and Land, 1990) after treatment with T4 DNA polymerase.

Growth of mutant HSV strains using complementing cell lines

A complementing cell line (B130/2) allowing growth of ICP27 deleted viruses and having no overlap between the complementing sequences and the ICP27 deleted viruses above (and thus preventing repair of ICP27 by homologous recombination during virus growth) was generated by co-transfection of plasmid pSG130BS (Sekulovich *et al.*, 1988) DNA with neomycin resistance-encoding plasmid pMamNeo (Invitrogen) into BHK cells and the selection of neomycin resistant clones. A clone highly permissive for the growth of an

HSV-1 ICP27 deletion mutant (B130/2) was selected for virus growth. PSG130BS carries a BamHI/SacI fragment from HSVL (nts 113322-115743) encoding the complete ICP27 coding sequence and part of UL55, but has no overlap with pΔMN.

There have been no previously reported ICP27 deletion mutant cell-line combinations in which there is no overlap between the sequences inserted into the cell-line for complementation and sequences remaining in the virus, and thus preventing repair of ICP27 by homologous recombination. Low level reversion to a pathogenic phenotype has previously been reported for ICP4 deletion mutant/ICP4 expressing cell line combinations in which there has been overlap between the remaining viral and inserted sequences, and thus allowing repair of the ICP4 mutation by homologous recombination.

Results

Male Lewis rats were stereotaxically inoculated in the striatum with 1 μl of a $10^9$ pfu/ml stock of either HSV strains zΔMN:16 or zΔMN:+ described above, or 1 μl of a $10^9$ pfu/ml of HSV-1 strain 1716/pR9. A drawn out glass capillary was used for inoculation and the inoculum was introduced over a period of 5 mins. 1716/pR9, which has been described previously is strain 1716 (deleted for ICP34.5) with a lacZ gene inserted into the non-essential gene UL43. After 2 days rats were killed and sections stained for lacZ activity with X-gal. X-gal positive cells were counted in each case.

| Virus | 1716/pR9 | zΔMN:16 | zΔMN:+ |
|---|---|---|---|
| Average number of blue cells/ 75 μm section (5 sections counted). | 12 | 680 | 740 |

These results show that while deletion of ICP34.5 does not preclude expression of a heterologous gene, this deletion alone does not allow highly efficient gene transfer, although it does provide a virus with severely reduced pathogenicity. However the additional removal of the essential IE gene ICP27, requiring virus growth on an ICP27 expressing cell line, not only provides increased safety, due to the absolute block to a productive infection in any cell

type in the absence of ICP27, and probable reduced cytotoxicity, it also highly significantly and unexpectedly increases the number of cells from which a heterologous gene can be expressed. Moreover this number of cells is not significantly reduced as compared to inoculation with a virus containing the ICP27 mutation alone (which is less safe because it is not deleted for ICP34.5).

The experiment using zΔMN: 16 was repeated using stereotaxic inoculation to the striatum of two 3 month old common marmosets (*Callithrix jacchus*) giving very similar results to the experiments rat experiments. Here greater than 600 cells, many with neuronal morphology, stained blue with X-gal in each 75 μm section. Thus the results obtained with rats have been extended to more relevant models of human disease, in this case with a species often used as a model system for studying Parkinson's disease.

These results clearly show that while deletion of ICP34.5 from the HSV genome provides a non-neurovirulent phenotype, the efficiency of heterologous gene expression of such a mutant in the brain is poor. However in combination with deletion of ICP27, ICP34.5/ICP27 deletion mutants unexpectedly provided highly efficient gene transfer to the brain. This, in combination with the anticipated advantage of a high degree of added safety and probable reduced cytotoxicity when using the double mutant, suggests that HSV-1 ICP34.5/ICP27 deletion mutants are highly promising for further development as vectors for gene delivery to the nervous system.

Example 2 - HSV strains defective in ICP34.5, ICP27 and VMW65

After infection by an ICP27/ICP34.5 double mutant, host cell metabolism will still be altered by the expression of a number of other HSV genes might be toxic *in vivo*. We thus further speculated that removal of further genes to minimise HSV gene expression would again improve the characteristics of the virus when used as a vector. To achieve this, an inactivating mutation was made in VMW65, the virion transcriptional activator protein, which is carried in the virion and is responsible for stimulating IE gene expression after infection (Ace *et al.*, 1989). This mutation should greatly reduce the levels of ICP0 and the other IE genes ICP22 and 47. These VMW65 mutants need not additionally be complemented in culture by a functional VMW65 gene as they are grown at high multiplicity or by inclusion of hexamethylene-bisacetamide (HMBA) in the media (McFarlane *et al.*, 1992).

Viruses

ICP34.5 deletion mutants with a mutation producing a functional inactivation of the transcriptional-activating activity of VMW65 were produced by co-cultivation (in BHK cells with 3 mM HMBA) of strain 1716, containing a deletion in both copies of ICP34.5 (MacLean *et al.*, 1991), with strain in1814 (Ace *et al.*, 1989) containing a functionally inactivated VMW65 gene. The genomic structure of resultant plaques was analysed by methods known to those skilled in the art (restriction digestion of purified genomic DNA and Southern blotting) and virus containing both the in1814 and 1716 mutations further plaque-purified five times, giving the virus strain 1764.

ICP27 was removed from strain 1764 using pΔMNlacZ and purified strain 1764 genomic DNA, as in Example 1 for the deletion of ICP27 from strain 1716, except with the inclusion of 3 mM HMBA in the media, giving strain zΔMN:64.

Results

HSV strain zΔMN:64 was tested both *in vitro* and *in vivo* as compared to the viruses described in Example 1. The results showed that, *in vivo*, similar numbers of blue X-gal staining cells were seen in brain sections 2 days after inoculating 1 x $10^6$ pfu stereotaxically into the striatum of a male Lewis rat as for zΔMN:+ and zΔMN:16 (approximately 700 blue staining cells/75 μm section). However while the level of gene transfer was relatively similar between the three viruses, the degree of macrophage infiltration as assessed by electron microscopy was somewhat reduced in zΔMN:64 as compared to zΔMN:+ and zΔMN: 16 suggesting a lower degree of cytotoxicity and/or immunogenicity, possibly associated with the reduced HSV gene expression when using zΔMN:64.

*In vitro* these differences were more marked as primary cultures of enteric neurons (derived from 7 day old Sprague-Dawley rat guts – Saffrey *et al.*, 1991) showed considerably enhanced survival (as assessed by trypan blue staining) and maintenance of neuronal morphology after 3 days in culture after treatment in a 96 well microtitre dish with 2 x $10^6$ pfu/well of zΔMN:64 as compared to zΔMN:+ or zΔMN:16. Thus 60-80% of cells maintained neuronal processes after 3 days with zΔMN:64 as compared to approximately 20% with zΔMN:+ or zΔMN:16. However even with zΔMN:64, after 7 days the number of cells

showing normal neuronal morphology was considerably reduced as compared to untreated cultures. Thus in this assay to assess toxicity zΔMN:64 showed enhanced characteristics as compared to zΔMN:+ and zΔMN:16 which also extends to the *in vivo* situation as described above.

Example 3 - HSV strains defective in ICP34.5, ICP27, VMW65 and ICP4.

However ICP4 levels are likely to be little reduced by the mutation in VMW65 and it is thus desirable to functionally inactivate ICP4 as well. Mutant HSV strains were therefore constructed that were additionally deleted for the ICP4 gene. Thus a further mutant HSV strain defective in ICP34.5, ICP27, ICP4 and VMW65 was produced. This strain should express only low levels of gene products, other than an inserted heterologous gene, in infected cells, as no HSV gene expression is stimulated by either VMW65, ICP27 or ICP4 and essentially none by ICP0 or ICP22.

Viruses

Both copies of ICP4 were removed from strain zΔMN:64 first by the removal of lacZ from the ICP27 locus. This was achieved by homologous recombination of purified strain zΔMN:64 genomic DNA with plasmid pΔMN into B1/30 cells, and selection of non-lacZ containing plaques by X-gal staining (white plaques) and Southern blotting, to confirm the removal of the entire lacZ gene, giving strain 1764/27$^{-}$w. Nucleotide numbers refer to the HSV strain 17+ sequence (Genbank no. HE1CG). A plasmid allowing deletion of ICP4 (pΔICP4) was then constructed using ICP4 flanking sequences (nts 123,459-126,774 [Sau3aI-Sau3aI] and nts 131,730-134-792 [SphI-KpnI] separated by XbaI and SalI sites derived from pSP72; Promega, in which the construct was made). An approximately 0.8 kb NotI fragment (nts 124,945-125,723) containing the coding region for ICP34.5 was also removed to prevent the repair of the ICP34.5 deletion during homologous recombination with 1764/27$^{-}$w.

A chimeric LAT promoter (nts 118,866-120,221 [PstI-BstXI]/CMV IE promoter (from pcDNA3 (Invitrogen))/lacZ (from pCH110; Pharmacia) cassette - the pR20 cassette - was then inserted at the unique XbaI site giving pΔICP4/pR20. This plasmid was then introduced into strain 1764/27$^{-}$w by homologous recombination as before and lacZ expressing plaques

identified by staining with X-gal and plaque purification on B4/27 cells (described below) complementing the mutations in both ICP4 and ICP27, giving virus strain 1764/27/4⁻/pR20. Plaque purified virus could not give a productive infection either on B 1/30 cells (expressing ICP27 but not ICP4) or BHK cells (expressing neither ICP27 nor ICP4).

ICP27/ICP4 double complementing cell line

A complementing cell line (B4/27) allowing growth of ICP27/ICP4 deleted viruses in which repair of the ICP27 or ICP4 deletions by homologous recombination is not possible was generated by co-transfection of plasmid pSG130BS (containing the ICP27 coding sequence and promoter; Sekulovich *et al.*, 1988) with pICP4 (a DdeI-SphI fragment [nts 126,764-131,730] containing the ICP4 coding region and promoter cloned between the EcoRV and SphI sites of pSP72 [Promega]) and pMamNeo (Invitrogen; encoding neomycin resistance) into BHK cells and selection of neomycin resistant clones. A clone highly permissive for growth of an ICP4 mutant and an ICP27 mutant was selected (B4/27).

There have been no previously reported ICP4/ICP27 deletion mutant/cell line combinations in which repair of the ICP4 or ICP27 by homologous recombination during virus growth is not possible.

Results

1764/27/4⁻/pR20 was tested *in vivo* as in Examples 1 and 2 and *in vitro* as in Example 2. *In vitro* results were similar to the results for zΔMN:64 except that neuronal morphology (presence of processes) was maintained until the end of the experiment (7 days), with no increase in cell death as compared to untreated controls. This demonstrated the reduced toxicity of this virus as compared to zΔMN:16 and zΔMN:64. These results suggest that these viruses are highly attenuated to a level such that effects on host cell physiology are minimal and suggest that any remaining toxicity *in vivo* is likely to be associated with immune responses to the virus particle after inoculation, and also immune responses to the high levels of expressed heterologous gene, in this case lacZ.

*In vivo* results in rats using 1 x $10^6$pfu of 1764/27/4⁻/pR20 inoculated as in Example 1, showed a similar number of cells transfected as with the other mutants zΔMN:+, zΔMN:16 and zΔMN:64 after 2 days, and from the *in vitro* data this can be expected to be associated

with further reduced toxicity. In this case this reduced toxicity and/or immunogenicity was demonstrated by improved expression characteristics in the longer term. Thus here, using the promoter/lacZ combination described (pR20), which is different to that used in Examples 1 and 2 but unlike the MMLV LTR promoter is active during herpes latency (unpublished results), approximately 30% of the number of cells which stain blue with X-gal after 2 days stain blue after 1 month. This compares to approximately 5% of cells staining blue after 1 month as compared to 2 days when using an ICP27-only deleted virus with the pR20 promoter/lacZ cassette inserted into the ICP27 locus.

Example 4 – HSV strains defective in *vhs*

Thus, for strains mutated in ICP34.5, ICP27, ICP4 and VMW65, cell physiology will only be altered by proteins delivered to the cell in the virion and possibly by expression of ICP6 which can still be expressed as an IE gene. Of these virion proteins, most are structural proteins and the virion host shutoff protein (*vhs*). *Vhs* is responsible for the destabilisation of host mRNAS in favour of translation from more rapidly produced HSV mRNAS after infection (Reef Hardy and Sandri-Goldin, 1994), and is thus potentially highly cytotoxic. Thus a further modification may be made to the mutant HSV strains of the invention by the functional activation of *vhs* (which is unnecessary for growth in culture).

*Vhs* mutations have not been included in vectors previously reported as having been tested for gene delivery *in vivo*. The ICP27 and ICP4 deletions give no overlap between the virus and the complementing sequences in the double-expressing cell line (i.e. expressing ICP27 and ICP4) used preventing any reversion by homologous recombination, and the ICP34.5 mutation provides a final level of safety in the unlikely event that the ICP27 and ICP4 deletions should become repaired.

References

Coffin RS, Latchman DS. Herpes simplex virus-based vectors. In: Latchman DS (ed). Genetic manipulation of the nervous system. Academic Press: London, 1996, pp 99-114.

MacLean AR *et al.* Herpes simplex virus type I deletion variants 1714 and 1716 pinpoint neurovirulence related sequences in Glasgow strain 17+ between immediate early gene I and the 'a' sequence. J Gen Virol 1991; 72: 632-639.

Shinnick TM *et al.* Nucleotide sequence of Moloney murine leukaemia virus. Nature 1981; 293: 543-548.

Morgenstern JP, Land H. A series of mammalian expression vectors and characterisation of their expression of a reporter gene in stably and transiently transfected cells. NAR 1990.; 18: 1068.

Sekulovich RE *et al.* J. Virol 1988; 64:3916-3926.

Ace C *et al.* Construction and characterisation of a herpes simplex virus type I mutant unable to transinduce immediate early gene expression. J Virol 1989; 63: 2260-2269.

McFarlane M, Daksis JI, Preston CM. Hexamethylene bisacetamide stimulates herpes-simplex virus immediate early gene-expression in the absence of trans-induction by VMW65. J Gen Virol 1992; 73: 285-292.

Schek N, Bachenheimer SL. Degradation of cellular mRNAs induced by a virion- associated factor during herpes simplex virus infection of Vero cells. J. Virol 1985; 55:601- 610

Rice, SA and Knipe DM. Genetic evidence for two distinct transactivation functions of the herpes simplex virus α protein ICP27. J. Virol 1990; 64: 1704-1715.

Reef Hardy, W and Sandri-Goldin RM. Herpes simplex virus inhibits host cell splicing and regulatory protein ICP27 is required for this effect J. Virol 1994; 68: 7790-7799.

Dobson, AT et al. A latent, non-pathogenic HSV-1-derived vector stably expresses β-galactosidase in mouse neurons. Neuron 1990; 5: 353-360.

Chou, J., Kern, ER, Whitley, RJ and Roizman, B. Mapping of herpes simplex virus-1 neurovirulence to $\gamma_1$34.5, a gene nonessential for growth in culture. Science 1990; 250: 1262-1266.

Chiocca, AE et al. Transfer and expression of the lacZ gene in rat brain neurons by herpes simplex virus mutants. New Biol. 1990; 2: 739-736.

Chou, J. and Roizman, B. The $\gamma_1$34.5 gene of herpes simplex virus 1 precludes neuroblastoma cells from triggering total shutoff of protein synthesis characteristic of programmed cell death in neuronal cells. PNAS 1992; 89: 3266-3270.

Chou, J., Poon, APW, Johnson, J. and Roizman B. Differential response of human cells to deletions and stop codons in the $\gamma_1$34.5 gene of herpes simplex virus. J. Virol. 1994; 68: 8304-8311.

Smith, IL, Hardwicke MA and Sandri-Goldin RM. Evidence that the herpes simplex virus immediate early protein ICP27 acts post-translationally during infection to regulate gene expression. Virology 1992; 186: 74-86.

Samaniego LA *et al.* J. Virol. 1995; 69: 5705-5715

DeLuca NA *et al.* J. Virol. 1985; 56: 558-570.

Lokensgard JR *et al.* J. Virol. 1994; 68: 7148-7158.

Saffrey *et al.*, Cell and Tissue Culture Research. 1991; 265: 527-534

## CLAIMS

1. A herpes simplex virus strain which lacks a functional ICP34.5 gene and a functional ICP27 gene.

2. An HSV strain according to claims 1 which is selected from a mutant HSV-1 strain, a mutant HSV-2 strain or derivatives thereof.

3. An HSV strain according to claim 1 or 2 wherein the lack of a functional ICP34.5 gene is due to a deletion or insertion within said gene.

4. An HSV strain according to any one of the preceding claims wherein the lack of a functional ICP27 gene is due to a deletion or insertion within said gene.

5. An HSV strain according to any one of the preceding claims which further lacks a functional second IE gene other than ICP27.

6. An HSV strain according to claim 5 wherein said IE gene is ICP4.

7. An HSV strain according to any one of the preceding claims which further lacks a functional VMW65 gene due to a mutation in said gene which abolishes its transcriptional-activation activity.

8. An HSV strain according to any one of the preceding claims which further lacks a functional *vhs* gene.

9. An HSV strain according to any one of the preceding claims which carries a heterologous gene.

10. An HSV strain according to claim 9 wherein said heterologous gene is operably linked to a control sequence permitting expression of said heterologous gene in mammalian cells.

11. An HSV strain according to claim 10 wherein said mammalian cell is a cell of the central or peripheral nervous system of a mammal.

12. An HSV strain according to claim 10 wherein said mammalian cell is a cell of the eye, heart or skeletal muscle of a mammal.

13. An HSV strain according to any one of claims 9 to 12 wherein said heterologous gene encodes a polypeptide of therapeutic use.

14. An HSV strain according to claim 13 wherein said gene encodes a polypeptide which is cytotoxic.

15. An HSV strain according to claim 13 wherein said gene encodes a polypeptide capable of converting a precursor prodrug into a cytotoxic compound.

16. An HSV strain according to any one of claims 10 to 13 wherein the heterologous gene is selected from genes encoding proteins involved in the regulation of cell division, enzymes involves in metabolic pathways, transcription factors and heat shock proteins.

17. An HSV strain according to claim 10 for use in delivering said heterologous gene to a mammalian cell.

18. An HSV strain according to any one of claims 10 to 17 for use in a method of treatment of the human or animal body.

19. An. HSV strain according to claim 18 for use in the treatment of disorders of, or injuries to, the nervous system of a mammal.

20. Use of a herpes simplex virus strain according to any one of claims 10 to 17 in the manufacture of a medicament for use in the treatment of the human or animal body.

21. Use of a herpes simplex virus strain according to claim 20 in the treatment of disorders of, or injuries to, the nervous system of a mammal.

22. A pharmaceutical composition comprising an HSV strain according to any one of claims 10 to 17 together with a pharmaceutically acceptable carrier or diluent.

23. A method for studying the function of a heterologous gene in a mammalian cell which method comprises:

(a) introducing said heterologous gene into an HSV strain according to any one of claims 1 to 8;

(b) introducing the resulting HSV strain into said mammalian cell; and

(c) determining the effect of expression of said heterologous gene in said mammalian cell.

24. A method according to claim 34 wherein said heterologous gene is a wild-type or mutant gene implicated in causing disease.

25. A method according to claim 23 or 24 wherein said mammalian cell is dysfunctional, said heterologous gene is wild-type and the effect of expression of said heterologous gene is determined by an assay for cellular function.

26. A method according to claim 23 or 24 wherein said mammalian cell has one or more endogenous genes inactivated by mutation.

27. A method for producing a herpes simplex virus according to claim 1 said method comprising modifying the ICP34.5 and ICP27 genes of a herpes simplex virus so as to functionally inactivate said genes.

28. A method according to claim 27 for producing a mutant HSV strain according to any one of claims 5 to 8, which method comprises modifying one or more IE genes and/or the VMW65 gene and/or the *vhs* gene of an HSV strain according to any one of claims 1 to 4.

29. A method of treatment of the human or animal body which method comprises administering an effective amount of a pharmaceutical composition according to claim 22 to a human or animal in need of such treatment.

30. A method according to claim 29 for use in the treatment of disorders of, or injuries to, the nervous system of a mammal.

31. A method of effecting gene therapy in a human or animal which method comprises introducing an HSV strain according to any one of claims 10 to 16 into the cells of a human or animal in need of such therapy resulting in effective expression of a heterologous gene encoding a therapeutic polypeptide in said cells.

# INTERNATIONAL SEARCH REPORT

Interr nal Application No
PCT/GB 97/02017

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/86 C12N7/04 C12N7/01 C07K14/035

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category ° | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | EP 0 453 242 A (GEN HOSPITAL CORP) 23 October 1991<br>see the whole document<br>--- | 1-7,9-31 |
| Y | WO 92 04050 A (ROIZMAN BERNARD) 19 March 1992<br>see the whole document<br>--- | 1-7,9-31 |
| Y | WO 96 04395 A (LYNXVALE LTD ;SPECK PETER GERALD (GB)) 15 February 1996<br>see the whole document<br>--- | 1-7,9-31 |
| | -/-- | |

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

3

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 1 December 1997 | 15 -12- 1997 |

| Name and mailing address of the ISA | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,<br>Fax: (+31-70) 340-3016 | Chambonnet, F |

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category ° | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | CHOU J ET AL: "MAPPING OF HERPES SIMPLEX VIRUS-1 NEUROVIRULENCE TO Y134.5, A GENE NONESSENTIAL FOR GROWTH IN CULTURE" SCIENCE, vol. 250, no. 4985, 30 November 1990, pages 1262-1266, XP000255907 see the whole document | 1-7,9-31 |

# INTERNATIONAL SEARCH REPORT

In. ..national application No.
PCT/GB 97/02017

**Box I Observations where c rtain laim were found unsearchable (Continuati n of item 1 f fir t sheet)**

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

see FURTHER INFORMATION sheet PCT/ISA/210

2. [X] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

see FURTHER INFORMATION sheet PCT/ISA/210

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

**Remark on Protest**

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

**FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

Claims Nos.: 24

because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

Claim 24 refers to " a method according to claim 34". But there is no claim 34. The search has been carried out considering it is a typing error and it must obviously be replaced by " a method according to claim 23".

Remark : Although claims 21,29-31 are directed to a method of treatment of the human/animal body , the search has been carried out and based on the alleged effects of the compound/composition. As far as claims 23-26 are directed to a diagnostic method practised on the human/animal body , the search has been carried out and based on the alleged effects of the compound/composition.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter nal Application No
PCT/GB 97/02017

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---|---|---|
| EP 0453242 A | 23-10-91 | AT 141517 T | 15-09-96 |
| | | CA 2039921 A | 17-10-91 |
| | | DE 69121452 D | 26-09-96 |
| | | DE 69121452 T | 02-01-97 |
| | | ES 2090243 T | 16-10-96 |
| | | JP 7095885 A | 11-04-95 |
| WO 9204050 A | 19-03-92 | US 5328688 A | 12-07-94 |
| | | AT 152355 T | 15-05-97 |
| | | AU 658838 B | 04-05-95 |
| | | AU 8741891 A | 30-03-92 |
| | | CA 2072627 A | 11-03-92 |
| | | DE 69125925 D | 05-06-97 |
| | | DE 69125925 T | 25-09-97 |
| | | EP 0500917 A | 02-09-92 |
| | | ES 2102409 T | 01-08-97 |
| | | JP 5503017 T | 27-05-93 |
| WO 9604395 A | 15-02-96 | AU 3119895 A | 04-03-96 |
| | | CA 2196168 A | 15-02-96 |
| | | EP 0772690 A | 14-05-97 |